IDENTIFICATION OF LIVESTOCK FAECAL CONTAMINATION IN SURFACE WATERS USING CHEMICAL AND MICROBIOLOGICAL TOOLS

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1 INTRODUCTION

Cattle and pig manure may contain pathogenic micro-organisms that can be transferred to soil through spreading on fields and thence to surface water. Such faecal pollution may pose risks to human health, especially in sensitive areas which support recreational shellfish harvesting, swimming and other uses where the public may come into direct contact with the water. Furthermore, the new European Directive on bathing waters (2006/7 /CE) requires the establishment of bathing water profiles, which require identification of sources of pollution. As the bacteria currently monitored to assess faecal pollution (*E. coli*, faecal coliforms and enterococci) do not distinguish between faecal pollution of water from animal and human sources, host-specific markers would be useful for the identification of faecal pollution. Recently, the concept of "Microbial source tracking" has been proposed (Scott *et al.,* 2002). It includes biological and chemical methodologies that could be used to identify the dominant sources of faecal contamination in surface waters. Among specific markers, steroids, intensity peaks and their ratios of three-dimensional fluorescence excitation−emission matrix (3D-EEM) spectroscopy, F+ RNA bacteriophages (FRNAPH) genotypes, host specific *Bacteroidales* and *Lactobacillus* appear to be interesting tools to distinguish human from animal faecal pollution (Leeming *et al*., 1996, Seurinck *et al*., 2005, Blanch *et al*., 2006; Mieszkin et *al.*, 2009, Naden *et al.*, 2009, Marti *et al.*, 2010). The aim of this study was to compare the suitability of chemical and microbiological markers to identify farm livestock sources of faecal contamination found in the environment. Four types of markers were tested: (i) the ratio of coprostanol/coprostanol+24-ethylcoprostanol (R1, expressed in percentage) and sitostanol/coprostanol (R2), (ii) tryptophan and fulvic-like fluorescence ratios (Bio/Geo and (V+VI/III)), (iii) genotypes of F-specific RNA bacteriophages (animal genotypes I and IV and human genotypes II and III) and (iv) bacterial markers belonging to *Bacteroidales* (human-specific HF183, ruminant-specific Rum-2-Bac and pig-specific Pig-2-Bac markers) and to the pig-specific *Lactobacillus amylovorus*.

2 MATERIALS AND METHODS

2.1 Sampling

Pig and bovine manure were collected from two farms located in Brittany (France). Six independent samples of field runoff water were collected after rainfall simulations on an experimental agricultural plot previously spread with either bovine or pig manure. Eight water samples were collected in Pays de la Loire (France). These include the effluents of three wastewater treatment plants (WWTP) and five samples, one of which was collected from a WWTP effluent (S1) and four (S0, S2, S3 and S4) from the Mayenne river (sample S0 was taken 50 m upstream of the WWTP effluent discharge, and three other samples taken downstream at distances of 10, 150 and 350 m, respectively). Three water samples were collected in the Daoulas river (Brittany, France) flowing through an agricultural area associated with intensive cattle pasture (B1 to B3); the distance between each point was approximately 500 m.

2.2 *E. coli* quantification

E. coli was counted using 3M^{TM} Petrifilm *E. coli* (3M, France) and TBX medium (OXOID, France) for samples of 1 mL and 100 mL volume, respectively. Inoculated media were incubated for 24 h at 44°C. Blue colonies (Glucuronidase positive) were counted to determine the concentration of *E. coli*.

2.3 Steroids

Steroid concentrations were determined using the method reported by Jardé *et al*. (2009). Briefly, freeze-dried samples were extracted by dichloromethane using an automated extractor (Dionex, ASE200). Hydrocarbons and polar molecules were separated into aliphatic hydrocarbons, aromatic hydrocarbons, and polar compounds by liquid chromatography on silica column using three successive elutions with organic solvents. Steroids compounds are present in the polar fraction. After derivatization using N,O-bis(trimethylsilyl)trifuoroacetamide (BSTFA) - trimethylchlorosilane (TMSC) (99/1, v/v), quantitative analyses of steroid compounds were performed on a Shimadzu QP2010+ gas chromatograph mass spectrometer. The quantification of steroid compounds was performed by the addition of $^{2}H_{6}$-cholestane as an internal standard in the polar fraction.

2.4 Fluorescence

Fluorescence measurements were performed using a Perkin-Elmer LS-55B luminescence spectrometer, with the protocol described by Bilal *et al*. (2010). A regional integration adapted from Chen *et al.* (2003) was applied on 5 mg.L^{-1} C-normalized solution. With this technique, EEM is divided into biochemical (Bio) (I, II, IV) and geochemical (Geo) (III, V) fluorescent regions and three peak intensity zones of tryptophan, fulvic like and humic like fluorescence. Data were normalized to a daily-determined water Raman intensity (excitation 350 nm, emission 395 nm, Raman emission intensity averaged 10.5 $^{+}$- 0.5 (n=10)). Two ratios were considered: Bio/Geo and V+III/VI.

2.5 *Bacteroidales* and *L. amylovorus*

The bacterial markers were quantified using real-time PCR. Volumes of 50 to 200 mL water were filtered on 0.22 µm or centrifuged at 9000 g for 15 min. Filters and pellets (250 mg) were transferred into microtubes and stored at -20°C. Genomic DNA of *Bacteroidales* and *L. amylovorus* were extracted using the Fast DNA Spin Kit for Soil and the QIAamp DNA Stool Kit, respectively. The primers used to amplify the Representational Difference Analysis fragment of the pig specific *L. amylovoru*s, and the 16S rRNA genes of HF183, Pig-2-Bac and Rum-2-Bac were described by Konstantinov *et al.* (2005), Seurinck *et al.* (2005) and Mieszkin *et al.* (2009, 2010), respectively.

2.6 F-specific RNA bacteriophages

FRNAPH were enumerated following the ISO method, either directly or after concentration by ultrafiltration Centricon Plus-70 (Millipore) for less contaminated water samples. Bacteriophage isolates were then genotyped by real time RT-PCR, with the One-Step RT-PCR kit, as in Ogorzaly *et al.* (2009).

3 RESULTS AND DISCUSSION

3.1 Values of the markers in manure, in runoff waters and in WWTP effluents

The values of the markers observed in manures, runoff waters contaminated by manure and in WWTP effluents are presented in table 1. Six of the markers appeared useful for differentiating livestock from human faecal pollution. The Pig-2-Bac and *L. amylovorus* markers were only detected in pig manure and runoff contaminated by pig manure whereas Rum-2-Bac and HF183 were specific to bovine and human contamination, respectively, confirming their host specificity reported by Seurinck *et al.* (2005), Mieszkin *et al.* (2009, 2010) and Marti *et al.* (2010). Steroids identified in pig manure were mainly represented by coprostanol, 24-ethylcoprostanol and cholestanol, whereas in bovine manure, the major steroids were 24-ethylepicoprostanol, sitostanol and sitosterol, in agreement with data of Leeming *et al.* (1996). As a consequence the coprostanol / coprostanol+ 24 ethylcoprostanol × 100 (R1 ratio) and sitostanol/coprostanol (R2 ratio) clearly differentiate bovine from pig contamination. Furthermore, the value of R1(> 65) and R2 (<0.3) in WWTP effluents also differentiates human from bovine pollution. The fluorescence ratios Bio/Geo and (V + III)/VI, which differed between pig and bovine manure, did not have the ability to distinguish the two types of pollution in water runoff samples. Animal genotypes (FRNAPH I and IV) were not detected in the pig manure and only FRNAPH I was detected in bovine manure with a low percentage of isolates (11.5%). These results agree with reports by Lee *et al.* (2009) who observed that only 25% of cow faeces

were positive for F specific coliphages. In the WWTP effluents, human genotypes (II+III) represented 67% of the isolates but animal genotypes were also detected (30.5% of isolates). As reported by Blanch *et al.* (2006), FRNAPH I and FRNAPH IV appeared less efficient for detecting animal contamination, due to their sporadic occurrence.

TABLE 1 **Mean concentrations (CFU, PFU, cells or copies / g or /100 mL) of microbial markers and chemical ratios in manures and waters contaminated by animal or human faecal pollution**

Sample type		*E. coli* (CFU)	Phages (PFU)	*L. amylovorus* (cells)	Pig-2-Bac (copies)	Rum-2-Bac (copies)	HF183 (copies)	Ratios R1	R2	Bio/Geo	(V+III)/VI
Pig manure	mean[a]	$3\ 10^4$	<lq[c]	$2.6\ 10^7$	$1.2\ 10^7$	<lq	<lq	59	0.23	0.47	1.33
(/g)	SD[b]	$7.1\ 10^3$		$1.2\ 10^7$	$1.3\ 10^5$			3	0.02	0.003	0.01
Bovine manure	mean	$5.1\ 10^4$	$1.2\ 10^2$	<lq	<lq	$2.2\ 10^7$	<lq	45	2.70	0.39	1.66
(/g)	SD	$4.5\ 10^4$				$5.6\ 10^6$		2	0.23	0.007	0.01
Runoff pig	mean	$1.4\ 10^4$	<lq	$3.5\ 10^7$	$6.8\ 10^5$	<lq	<lq	57	0.62	0.25	1.35
(/100 mL)	SD	$6.7\ 10^3$		$1.4\ 10^7$	$3.4\ 10^5$			1	0.01	0.06	0.06
Runoff bovine	mean	$3.4\ 10^5$	$1.1\ 10^3$	<lq	<lq	$1.1\ 10^7$	<lq	49	2.18	0.24	1.22
(/100 mL)	SD	$6.6\ 10^5$				$7.2\ 10^6$		1	0.03	0.02	0.15
WWTP	mean	$3.1\ 10^5$	$7.4\ 10^3$	<lq	<lq	<lq	$3.1\ 10^6$	70	0.26	0.22	0.88
(/100 mL)	SD	$2.9\ 10^5$	$8.4\ 10^3$				$3.1\ 10^6$	8	0.25	0.034	0.06

[a] mean of three values; [b] standard deviation ; [c] limit of quantification

3.2 Values of markers in rivers impacted by human or bovine contamination

In the Mayenne river, impacted by a discharge of a WWTP effluent (S0 to S4 samples), none of the animal specific bacterial markers were found in samples upstream or downstream of the effluent discharge (table 2). Human-specific marker HF183 and FRNAPH were detected only in the most contaminated samples (S1 and S2). The human genotypes (II and III) represented 79% and 95% of the isolates in the water samples S1 and S2, respectively. The values of R1 and R2, which were specific to human pollution in the WWTP effluent, were less informative in river samples, probably due to the lower contamination (concentration of *E. coli* $<10^3$ CFU/100 mL). As the Mayenne river is located in a rural area, the presence of *E. coli* could be the result of a mixed bovine and human faecal contamination, which was too weak to be quantifiable using the real-time PCR. Conversely, the origin of contamination in the river flowing through intensive cattle pasture (B1 to B3 samples, table 2) was clearly identified by Rum-2 Bac, the absence of HF 183, Pig-2-Bac and *L. amylovorus* markers, low values of the ratio R1 (<44) and the high values of the ratio R2 (>1.3). Two of the markers failed to indicate this bovine contamination despite a high level of *E. coli*: FRNAPH (not detected) and the Bio/ Geo and (V+III)/VI ratios (uninterpretable).

TABLE 2 **Mean concentrations (CFU, PFU, cells or copies / 100 mL) of microbial markers and chemical ratios in two rivers, impacted either by a treated urban effluent (Mayenne river) or by bovine contamination (Daoulas river)**

Origin of pollution	sample	*E. coli* (CFU)	Phages (PFU)	*L. amy*[a] (cells)	Pig-2-Bac (copies)	Rum-2-Bac (copies)	HF183 (copies)	Ratios R1	R2	Bio/Geo	(V+III)/VI
Human	S0 (upstream)	$5\ 10^1$	<lq[b]	<lq	<lq	<lq	<LQ	56	0.76	0.11	1.16
	S1 (WWTP effluent)	$6.3\ 10^5$	$1{,}70\ 10^4$	<lq	<lq	<lq	$4.4\ 10^4$	70	0.24	0.24	0.94
	S2 (discharge)	$3.2\ 10^3$	320	<lq	<lq	<lq	$8.1\ 10^4$	nd[c]	1.01	0.11	1.15
	S3 (downstream)	$5\ 10^2$	<lq	<lq	<lq	<lq	<lq	51	0.96	0.11	1.15
	S4 (downstream)	$3.3\ 10^2$	<lq	<lq	<lq	<lq	<lq	54	0.86	0.11	1.17
Bovine	B1	$1.9\ 10^4$	<lq	<lq	<lq	$2.5\ 10^5$	<lq	43	1.4	0.07	1.0
	B2	$1.5\ 10^4$	<lq	<lq	<lq	$1.3\ 10^5$	<lq	39	1.8	0.07	1.0
	B3	$1.7\ 10^4$	<lq	<lq	<lq	$1.6\ 10^5$	<lq	38	1.8	0.07	1.0

[a] *L. amylovorus*; [b] limit of quantification; [c] no data

4 CONCLUSIONS

In conclusion, as previously reported by Blanch *et al.* (2006), our study confirmed that the identification of the source of faecal contamination is improved by the use of several markers. Although contamination sources in the Mayenne river were more difficult to identify due to the low level of *E. coli* and the possibility of mixed contamination (bovine and human), highly contaminated samples (runoff waters contaminated by manure, Daoulas river) could be easily characterised with most of the animal markers: steroid ratios R1 and R2, and bacterial markers Rum-2-Bac, Pig-2-Bac and *L. amylovorus*. The suitability of the proposed markers has been demonstrated by their transfer via runoff to surface waters and their detection in water contaminated by bovine faeces.

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